PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/86 A2

(11) International Publication Number: WO 99/10746

(43) International Publication Date: 4 March 1999 (04.03.99)

(21) International Application Number: PCT/US98/17434

(22) International Filing Date: 25 August 1998 (25.08.98)

(30) Priority Data:
08/920,151 26 August 1997 (26.08.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US Not furnished (CON)
Filed on Not furnished

(71) Applicant (*for all designated States except US*): THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; 308 Bynum Hall, CB #4105, Chapel Hill, NC 27599-4105 (US).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): WU, Hai-feng [CN/US]; 101 William White Court, Carrboro, NC 27510 (US).

(74) Agents: SIBLEY, Kenneth, D. et al.; Myers, Bigel, Sibley, & Sajovec, P.A., P.O. Box 37428, Raleigh, NC 27627 (US).

(81) Designated States: AU, CA, JP, KR, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD OF MONITORING BLOOD LOW MOLECULAR WEIGHT HEPARIN AND HEPARIN

(57) Abstract

A method for determining the concentration of low molecular weight heparin or heparin in a blood plasma sample comprises the steps of: (a) adding a dilute thromboplastin (or tissue factor) solution to a blood plasma sample; and then (b) measuring the time to clot formation in said blood plasma sample. In one embodiment, the dilute thromboplastin (or tissue factor) solution is diluted prior to the adding step so that the time to clot formation in said blood plasma sample is at least 100 seconds when said blood plasma sample contains at least 1 μg/ml of low molecular weight heparin.

METHOD OF MONITORING BLOOD LOW MOLECULAR WEIGHT HEPARIN AND HEPARIN

Field of the Invention

The present invention relates to prothrombin time assays, and particularly relates to modified prothrombin time assays that are capable of measuring the concentration of heparin and heparin derivatives in blood.

Background of the Invention

Heparin is a heavily sulfated glycosaminoglycan obtained by extraction from animal lung or intestine (B. Casu, Heparin Structure, *Haemostasis* (1990)). It contains heterogeneous polysaccharide chains with a mean molecular weight of 12.000 to 16,000 daltons (R. Rosenberg et al., *Proc. Natl. Acad. Sci. USA* **75**, 3065 (1978)). Heparin functions as a catalytic cofactor for an endogenous antithrombin and heparin cofactor II (M. Bourin et al., *Biochem. J.* **289**, 313 (1993); C. Jackson, *Baillieres Clin. Haematol.* **3**, 483 (1990); C. Hemker et al., *Adv. Exp. Med. Biol.* **313**, 221 (1992); B. Bray et al., *Biochem. J.* **262**, 225 (1989)).

The interaction of heparin with antithrombin or heparin cofactor II can effectively inactivate many of the proteases involved in blood coagulation. Heparin has been used widely as an anticoagulant drug for hemostatic disorders, including deep vein thrombosis, pulmonary embolism, myocardial infarction and disseminated intravascular coagulation (M. Gibaldi et al., *J. Clin. Pharmacol.* **35**, 1031 (1995)). Additionally, heparin is used prophylactically in clinical procedures such as cardiovascular surgery, orthopedic surgery and hemodialysis to prevent possible thrombosis. The heterogeneous nature of heparin is probably the reason that its clinical use is associated with side effects such as hemorrhage and thrombocytopenia. Accordingly, Low molecular weight (LMW) heparins have been developed as a safer alternative.

LMW heparin contains a relatively homogenous composition of glycosaminoglycan chains with an average molecular weight of about 5,000 daltons (J.

Fareed et al., *Seminars in thrombosis and Hemostasis,* **19 suppl. 1:** 1 (1993); W. Jeske et al., *Seminar Thromb. Hemost.* **19**, 229 (1993)). LMW heparin has fewer hemorrhagic side effects than regular heparin (M. Freemen, *J. Clin. Pharmacol.* **31,** 298 (1991)). Additionally, LMW heparin has a better bioavailabilty, with a biological half life of 3 to 4 hours. The half life for regular heparin is only about 30 minutes (A. Mewborn et al., *Am. J. Health-syst. Pharm.* **53,** 167 (1996)). Therefore, LMW heparin is clinically replacing standard heparin for a number of clinical applications (J. Fareed et al., *supra*). Three forms of LMW heparin that are currently approved by FDA are enoxaparin (Lovenox™), dalteparin (Fragmin™) and danaparoid (Orgaran™).

The clinical use of regular heparin must be monitored to prevent hemorrhage. Standard heparin is monitored by an activated partial thromboplastin time (aPTT) assay (*see, e.g.*, U.S. Patents Nos. 4,672,030 and 5,506,146), which can detect blood heparin activity as low as 1 ug/mL. Although LMW heparin is associated with less tendency of hemorrhage, monitoring therapeutic use of LMW heparin has been recommended, particularly in the patients with renal dysfunction (M. Samama, *Thromb. Hemost.* **15,** 119 (1995); J. Walenga, *Seminars in Thrombosis and Hemostasis* **19 suppl. 1,** 69 (1993)). Significantly, the standard antagonist used to neutralize excessive heparin, protamine sulfate, is not an effective antagonist for LMW heparin. No other specific LMW heparin antagonists have yet been developed.

Since there is no pharmacologically effective antidote for LMW heparin, higher dosages of LMW heparin used therapeutically must be monitored effectively to prevent catastrophic hemorrhage. The issue of whether prophylactic dosages of LMW heparin should also be monitored has been debated over the last several years (P. Bacher et al., *Seminar Thromb. Hemost.* **1993**, 73 (1993)).

Two methodologies have been developed to monitor blood LMW heparin activity: the HEPTEST™ assay and the anti-Xa assay (M. Samama, *supra*; E. Yin, *Biochem. biophys. Acta* **201**, 387 (1969); T. Ozawa et al., *Thromb. Res.* **66,** 278 (1992)). The HEPTEST™ is a clot based assay, while the Anti-Xa assay is an enzymatic assay. Both assays require addition of purified bovine Factor Xa to the assay system and are therefore expensive. The anti Xa assay uses a chromogenic substrate and also requires special equipment and expertise.

The Prothrombin time (PT) assay is the most often used clotting assay in the clinical laboratory. The PT assay is used for the diagnosis of many hereditary coagulation disorders, including factor II, V, VII, X, and fibrinogen deficiencies. It is also used to monitor hemostatic status for the patients receiving the anticoagulant drug warfarin. Unfortunately, the standard PT assay is not sensitive to heparin or LMW heparin (*see, e.g.,* Organon Teknica Corp. Simplastin® Excell (October 1988)(product insert instruction sheet).

Summary of the Invention

The present invention provides a modified prothrombin time assay which is sensitive to the blood level of LMW heparin and heparin. Additionally, both relipidated and nonlipidated tissue factor can be used to initiate the blood clotting assay, which is also sensitive to the blood level of LMW heparin and heparin.

A first aspect of the present invention is, accordingly, a method for determining the concentration of LMW heparin in a blood plasma sample. The method comprises the steps of (a) adding a diluted thromboplastin solution to a blood plasma sample; (b) measuring the time to clot formation in said blood plasma sample; (c) using the foregoing method to generate a standard curve with control plasma which contain a range of known concentration of LMW heparin; and then (d) extrapolating (calculating) the concentration of LMW heparin in said blood plasma sample based on the standard curve. The method is further defined in that the dilute thromboplastin is diluted prior to the adding step so that the time to clut formation is at least 60 seconds when plasma is free of LMW heparin and the time to clot formation is at least 100 seconds when the plasma contains at least 1 ug/mL (or 0.1 units/mL) of LMW heparin (*e.g.,* 8 ug/mL of enoxaparin).

A second aspect of the present invention is a method for determining the concentration of LMW heparin in a blood plasma sample. The method comprises the steps of : (a) adding a recombinant tissue factor solution to a blood plasma sample; (b) measuring the time to clot formation in said blood plasma sample: (c) using the above method to generate a standard curve with control plasma which contains a range of known concentration of the LMW heparin; and then (d) extrapolating (calculating) the concentration of LMW heparin in said blood plasma sample based on the standard curve.

The method is further defined in that the dilute recombinant tissue factor solution is diluted prior to the adding step so that the time to clot formation is at least 60 seconds when the plasma is free of LMW heparin and the time to clot formation is at least 100 seconds when the plasma contains at least 1 ug/mL (or 0.1 units/mL) of LMW heparin (*e.g.*, 8 ug/mL of enoxaparin).

A third aspect of the invention is a method for determing the concentration of regular, or standard, heparin in a blood plasma sample. The method comprises the steps of (a) adding either a diluted thromboplastin solution or adding a recombinant tissue factor solution to a blood plasma sample; (b) measuring the time to clot formation in said blood plasma sample; (c) using the above method to generate a standard curve with control plasma which contains a range of known concentrations of heparin; and then (d) extrapolating (calculating) the concentration of heparin in said blood plasma sample based on the standard curve. The method is further defined in that the dilute thromboplastin or recombinant tissue factor solution is diluted prior to the adding step so that the time to clot formation is at least 60 seconds when the plasma is free of LMW heparin and the time to clot formation is at least 100 seconds when the plasma contains at least 1 ug/mL (or 0.1 units/mL) of LMW heparin (*e.g.*, 8 ug/mL of enoxaparin).

A fourth aspect of the invention is kits useful for carrying out the methods described above.

The foregoing and other objects and aspects of the present invention are explained in detail in the specification herein and the drawings described below.

Brief Description of the Drawings

Figure 1 shows the effects of thromboplastin concentration (folds of dilution) on clotting time in seconds for various sources of thromboplastin. The top panel is for Sigma thromboplastin; the center panel is for Ortho Diagnostics thromboplastin; the bottom panel is for Organon Teknica thromboplastin. The left bar in each pair of bars is for control plasma, and the right bar is for plasma that contains added enoxaparin.

Figure 2A shows the clotting time in seconds for various concentrations of enoxaparin in plasma, as measured by a fibrometer (circles) and a microplate (triangles).

Figure 2B shows the ratio of clotting time (RCT) for various concentrations of

enoxaparin in plasma, as measured by a fibrometer (circles) and a microplate (triangles).

Figure 3A shows the ratio of clotting time for various concentrations of enoxaparin (μg/mL) in plasma, as measured by a microplate.

Figure 3B shows the ratio of clotting time for various concentrations of dalteparin (μg/mL) in plasma, as measured by a microplate.

Figure 3C shows the ratio of clotting time for various concentrations of danaparoid (μg/mL) in plasma, as measured by a microplate.

Figure 4 shows the ratio of clotting time for various concentrations of heparin derivatives (μg/mL) (Squares: Mr.=6000; triangles: Mr.=3000; circles: Mr.=3700).

Figure 5 shows the ratio of clotting time for various concentrations (μg/mL) of glycosaminoglycans (Squares: heparan sulfate; Circles: chondroitin sulfate E; triangles: dermatan sulfate; circles with +: heparin).

Figure 6 shows the clotting time in seconds for the assays indicated by adding various concentrations (ng/mL) of recombinant tissue factor. Circles represent relipidated tissue factor; triangles represent nonlipidated tissue factor.

Figure 7 shows the clotting time in seconds for various concentrations of heparin derivatives. Circles represent enoxaparin; triangles represent heparin derivative (Mr.=3000).

The present invention is explained in greater detail in the specification below.

Detailed Description of the Invention

The present invention provides a method for determining the concentration of low molecular weight heparin or heparin in a blood plasma sample. The term "low molecular weight heparin" herein has its usual meaning, and refers to a variety of heparin-derived glycosaminoglycan products obtained from heparin by fractionation, depolymerization, digestion, etc., that are classified together in the art. *See, e.g.*, M. Freedman, *Low Molecular Weight Heparins: An Emerging New Class of Glycosaminoglycan Antithrombotics*, J. Clin. Pharmacol. 31, 298 (1991); J. Fareed et al., *Current Perspectives on Low Molecular Weight Heparins*, Seminars in Thrombosis and Hemostasis **19 suppl. 1**, 1-11 (1993); M. Samana, *Contemporary Laboratory Monitoring of Low Molecular Weight Heparins*, Clinics in Laboratory Medicine **15**, 119-123 (March

1995). Furthermore, the invention also provides a method for determining the concentration of other glycosaminoglycans such as dermatan sulfate, chondroitin sulfate or heparan sulfate in a blood plasma sample.

The method is a modified prothrombin time (PT) test, which may advantageously be carried out in much the same manner as conventional prothrombin time tests. The method solves the problem of previous prothrombin time tests, which (as noted above) cannot be used to monitor heparin therapy. In overview, the method comprises adding a dilute thromboplastin solution to a blood plasma sample; and then measuring the time to clot formation in the blood plasma sample. The time to clot formation indicates the quantity of heparin or low molecular weight heparin in the sample, with longer times indicating a larger quantity of heparin or low molecular weight heparin in the sample. The measuring step may be followed by the step of determining the quantity of heparin or LMW heparin in the sample, with quantity being determined by techniques known in the art, such as preparing a set of serially diluted standards against which the unknown sample is measured.

To make the detection of heparin or LMW heparin possible, the thromboplastin solution must be diluted to a greater extent than suggested in prior PT assays. In general, the dilute thromboplastin solution is diluted prior to the adding step so that the time to clot formation in said blood plasma sample is at least 100 seconds, and more preferably at least 200 seconds, (up to about 800 or 1000 seconds) when said blood plasma sample contains a therapeutic level of LMW heparin (*e.g.*, 8 μg/ml enoxaparin). Stated alternatively, the thromboplastin solution should be diluted prior to the adding step so that the ratio of clotting time (the clotting time in the presence of the said quantity of enoxaparin divided by the clotting time in the absence of the said quantity of LMW heparin) is at least 1.5 (up to 2, 3, 4, or 5). The standard assay for determining these times and ratios for the dilute thromboplastin solution may be carried out in accordance with known techniques, including fibrometer, automated clotting assay or microplate assay (*e.g.*, by mixing 40μL of blood plasma anticoagulated with sodium citrate in a microplate well with 8μL saline containing the said quantity of enoxaparin and incubating at 37°C for 3 minutes, and then adding 72 μL prewarmed (37°C) diluted thromboplastin reagent in 25 mM $CaCl_2$ to start the clotting assay).

Other conditions of the assay are known to those skilled in the art of PT assays. In general, an anticoagulant such as sodium citrate is added to the blood plasma sample. The thromboplastin solution contains at least 3mM calcium ions, and typically contains 8 or 9 to 30 or 40 mM calcium ion (usually in the form of added $CaCl_2$). The thromboplastin solution is typically buffered with HEPES, TAPSO, MOPS, TES, DIPSO, POPSO, TRIS (these abbreviations having their standard meaning in the art, *see e.g.*, U.S. Patent No. 5,625,036)or others, in a concentration, for example, of 20 to 80 mM. From 0 to 300 mM NaCl may be included. The thromboplastin solution and the blood plasma sample are preferably warmed to a temperature of about 35 to 40 degrees centigrade, and most preferably to 37°C, prior to said adding step.

The assay may be carried out by hand or may be automated, and may be carried out with any suitable laboratory equipment. For example, the assay may be carried out with a fibrometer, any commercially available automated clotting machine, or as a microplate assay, in accordance with known techniques.

The materials for carrying out the method described above may be conveniently provided in the form of a kit. Such a kit typically contains *(a)* a concentrated thromboplastin reagent (provided as a dehydrated, freeze dried or lyopholized powder, or as a concentrated solution); and *(b)* a diluent for the concentrated thromboplastin reagent (provided as a dehydrated, freeze dried or lyopholized powder, or as a diluted solution). The diluent may contain stabilizers and preservatives as is known in the art. The thromboplastin reagent may be produced from any suitable source such as rabbit brain, as is known in the art. One preferred thromboplastin reagent for carrying out the present invention, as explained in greater detail in the examples below, is Simplastin®Excel, manufactured by Organon Teknica Corp., Box 15969, Durham, North Carolina, USA 27704-0969 (product numbers 52000 or 52001). The kit preferably includes instructions (*e.g.*, printed instructions) for combining the diluent and the concentrated thromboplastin reagent to provide a dilute thromboplastin solution that yields a time to clot formation as described above. Typically, sufficient diluent is provided for a predetermined number of assays (*e.g.*, 300 tests, 500 tests; typically 100 to 2000 tests), and sufficient concentrated thromboplastin solution is provided for the given number of assays.

As noted above, also disclosed herein is a method for determining the concentration of heparin or low molecular weight heparin in a blood plasma sample with a recombinant tissue factor (*i.e.*, recombinant thromboplastin). In general, the method comprises *(a)* adding a dilute (or appropriate) recombinant tissue factor solution to a blood plasma sample so that the time to clot formation is at least 100 seconds; and then *(b)* measuring the time to clot formation in blood plasma sample. Again, the measuring step may be followed by the step of determining the quantity of heparin or LMW heparin in the sample, with quantity being determined by techniques known in the art, such as preparing a set of serially diluted standards against which the unknown sample is measured.

The recombinant tissue factor may be a complete tissue factor protein or an active fragment thereof (*e.g.*, the cytoplasmic portion of the tissue factor). The tissue factor may be of any suitable species of origin, such as rabbit or human, but is typically of mammalian origin. The recombinant tissue factor formulation preferably is a lipidated formulation: that is, it includes one or more phospholipids (*e.g.*, phosphatidylcholines, phosphatidylserines) in an amount sufficient to activate the recombinant tissue factor (*e.g.*, at least one phospholipid that has an unsaturated fatty acid side chain). The protein may be associated with the lipid in any suitable structure, such as a liposome or a micelle. One example of such a recombinant tissue factor formulation is described in U.S. Patent No. 5,625,036, the disclosure of which is incorporated herein by reference. The dilute recombinant tissue factor solution is diluted prior to said adding step so that the time to clot formation in said blood plasma sample is at least 100 seconds, and more preferably at least 200 seconds (up to about 700 or 1000 seconds) when the blood plasma sample contains a therapeutic level of LMW heparin (*e.g.*, at least 1 ug/mL (or 0.1 units/mL) of LMW heparin, for example 8 μg/ml enoxaparin) in a standard assay as described above.

Other conditions for carrying out assays with recombinant tissue factor, and kits for carrying out such assays, are essentially the same as described above in connection with natural thromboplastin solutions, with the recombinant tissue factor substituted for the natural thromboplastin.

Methods and kits of the present invention can be used to measure both heparin and LMW heparin in blood plasma. Advantageously, the methods and kits of the present

invention are not inconsistent with other tests performed with the PT assay, such as deficiencies in the extrinsic coagulation system (Factor II, V, VII and X), fibrinogen deficiencies, and for monitoring oral anticoagulant therapy. This enables a greater variety of tests to be performed with a smaller number of procedures and reagents.

The present invention is explained in greater detail in the following non-limiting examples. All chemicals used were from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated, and were of the highest grade purity available. PT reagents were purchased from Sigma, Ortho Diagnostics and Organon Teknika, respectively. Reference normal plasma was obtained from American Diagnostica Inc. (Greenwich, CT); aPTT reagents were obtained from Pacific Hemostasis (Ventura, CA); heparin with an average molecular weight of 15 KD was provided by Diosynth (Oss, the Netherlands); Heparan sulfate was obtained from Enzyme Research Lab., Inc.; Dermatan sulfate was from Calbiochem. Chondroitin sulfate E was purchased from Seikagaku America, Inc.; Lovenox was purchased from UNC hospital pharmacy. Fragmin™ and Orgaran™ were kindly provided from Pharmacia Inc. and Organon Inc., respectively.

EXAMPLE 1

Prothrombin Time Assay Formats

1. Clotting assay by Fibrometer. A clotting assay with a Fibrometer was performed according to standard protocol in the hospital laboratory. Briefly, 20 μL saline containing a known concentration of LMW heparin was added to 100 μL plasma. The sample was mixed and incubated at 37°C for 3 minutes. A 180 μL prewarmed (37°C) diluted thromboplastin solution was then added and clotting time was started immediately.

2. Microplate clotting assay. The clotting assays were performed in a microplate as described previously (C. Pratt et al., *BioTechniques* **13**, 430 (1992); H. Wu, *Blood* **85**, 421 (1995)). The following procedure was used unless specified in the text. 40 μL normal reference plasma (anticoagulated with sodium citrate) in each well was mixed and incubated with 8 μL saline containing the indicated reagent at 37°C for 3 minutes. 72 μL prewarmed (37°C) diluted thromboplastin reagent in 25 mM $CaCl_2$ was then added to start clotting assay. The clotting time was determined kinetically by onset OD defined

by 0.02 at 405 nM, as detailed previously (J. Harenbert et al., *Haemostasis* **19,** (1989)). The clotting time represents the time elapsed from adding the reagent (thromboplastin) until an increase of 0.02 at 405 nM is achieved (F. Dati et al., *Thromb. Hemost.* **58,** 856 (1987)). A ratio of clotting time (RCT) is calculated as the clotting time in the presence of heparin derivative divided by clotting time in the absence of heparin derivative. RCT thus represents the drug activity illustrated by a ratio of clotting time.

When recombinant tissue factor was used to initiate clotting assays instead of thromboplastin reagents, 40 μL plasma was first mixed and incubated with 8 μL reagent at indicated concentration. The clotting time was started by adding 72 μL tissue factor in 25 mM $CaCl_2$.

Calcium concentration in the clotting reaction was kept constant at 15 mM. Preliminary data with titration of calcium concentration have shown that the clotting time was not affected by calcium when its concentration in the reaction was above 3 mM.

All the assays in this study were done in either triplicate or duplicate. Mean values and standard deviations (S.D.) were determined using Cricket Graph version III on a Power Macintosh computer. Standard deviation was less than 5% of the mean value for the most of the values and was not visible in the plot.

EXAMPLE 2

The Effect of Various Dilutions of Thromboplastin on Enoxaparin-Mediated Prolongation of Clotting Time

Various concentrations of thromboplastin diluted in 25 Mm $CaCl_2$ were used to initiate clotting assays in the microplate. Thromboplastin preparations obtained from different sources were also evaluated. The blood clotting times were compared in both absence or presence of enoxaparin (8 ug/Ml) added to plasma. It was shown that as the concentration of thromboplastin was diluted, enoxaparin-mediated prolongation of clotting time became more and more significant (**Figure 1**). When the concentration of thromboplastin is diluted from 1:200 to 1:6400, the clotting time in the absence of enoxaparin is prolonged from less than 100 seconds to 240 seconds, while the clotting time in the presence of enoxaparin is prolonged from less than 150 seconds to 750 seconds. The ratio of clotting time reach 2 when thromboplastin is 1600 times diluted

and is greater when thromboplastin is further diluted. All three preparations of thromboplastin tested have similar results and they are all sensitive to enoxaparin when diluted preparation was used. I arbitrarily selected a concentration of 3000 fold thromboplastin dilution and chose the preparation from Sigma for the rest of the experiments.

EXAMPLE 3

Comparison of Clotting Assays by Fibrometer and by Microplate Densitometry

Fibrometers have been used as a standard device to measure PT and aPTT in the hospital laboratory. Microplate densitometry assays were recently developed, and appear to be as valid as the Fibrometer for measuring PT and aPTT. The fibrometer assay measures the resistance of a solution as the result of clot formation, while the microplate densitometry assay measures the change of optical density in the solution by clot formation. Thromboplastin (Sigma) at 1:3000 dilution was used to initiate clotting assays. The results obtained by the fibrometer ware compared to those obtained by microplate densitometry. The clotting time was plotted against various concentration of enoxaparin in plasma (**Figure 2A**). The same data was also plotted by the ration of clotting time against the concentration of enoxaparin in plasma (**Figure 2B**). Both assays demonstrate that the clotting time was significantly prolonged when the concentration of enoxaparin in the plasma is higher than 1 μg/mL.

EXAMPLE 4

Dose-Dependent Prolongation of Clotting Time on Three Different Forms of LMW Heparin

The sensitivity of the diluted PT assay to three different forms of LMW heparins (enoxaparin (Lovenox), dalteparin (Fragmin) and danaparoid (Orgaran)) was also examined. Various concentration of LMW heparin were preincubated with plasma for 5 minutes at 37°C before the addition of thromboplastin (at 1:3000 dilution) to initiate clotting assays. The ratio of clotting time was plotted against the concentration of each LMW heparin in plasma. As shown in **Figure 3**, the assay was almost equally sensitive to all three forms of LMW heparin. In addition, there is a linear relationship between the plasma concentration of LMW heparin and the ratio of clotting time in the range between 1 to 10 μg/mL for enoxaparin, between 0.1 to 0.75 units/mL for dalteparin and between 0.1 to 1 units for danaparoid, respectively. It has been reported that the plasma concentration of LMW heparin in patients on therapeutic dosage are 2 - 6 μg/mL for enoxaparin, or 0.2 - 0.6 units/mL for dalteparoid and danaparoid respectively (J. Albada, *Circulation* 80, 935 (1989); M. Verstraete, *Drugs* 40, 498 (1990)). This assay is therefore able to quantitate LMW heparin activity in patient blood. The concentration of LMW heparin in patient blood can be extrapolated (estimated) based on standard curves obtained, as shown in **Figure 3**.

EXAMPLE 5

Dose-Dependent Prolongation of Clotting Time on Various Molecular Weights of Heparin Derivatives

Although heparin and LMW heparin are effective anticoagulant drugs, many companies are still trying to develop new heparin derivatives as an anticoagulant drug. These possible candidates would either have a different molecular weight or have a different structure of the glycan backbone comparing to standard heparin drug. Accordingly, the sensitivity of this assay to heparin derivatives with different molecular weights was examined. As seen in **Figure 4**, the assay was extremely sensitive to regular heparin in blood. When heparin with a molecular weight of 3,000 daltons was added to plasma, the clotting time was also significantly prolonged. Therefore, this assay also can

be used to evaluate even smaller fragments of heparin in blood plasma samples.

EXAMPLE 6

Dose-Dependent Prolongation of Clotting Time on Different Forms of Glycosaminoglycan Derivatives

The ability of this assay to evaluate the activity of various glycosaminoglycan derivatives in blood was also examined. Four different glycosaminoglycans (heparin, dermatan sulfate, heparan sulfate and Chondroitin sulfate) were included in this study. It was shown in **Figure 5** that the assay was sensitive to blood levels of heparin and dermatan sulfate, but is less sensitive to blood levels of Chondroitin sulfate E and heparin sulfate. Dermatan sulfate is a specific glycosaminoglycan which only accelerates the thrombin/heparin cofactor II reaction, and it is being developed as an alternative anitcoagulant drug. Heparan sulfate and Chondroitin sulfate E are potential candidates as anticoagulant drugs.

EXAMPLE 7

Clotting Assays Initiated by Recombinant Tissue Factor

Thromboplastin used in the clotting assay is extracted from animal tissue and is a partially purified form of tissue factor. Recombinant tissue factor is now available. Recombinant tissue factor was generated by mammalian cell line and the relipidated form of tissue factor can then be obtained by chemical modification. The lipid moiety of tissue factor is essential for effective expression of tissue factor activity and, therefore, the relipidated form has much higher activity (Y. Nemerson, *Thromb. Hemost.* 74, 180 (1995)). Both non-lipidated and relipidated forms of recombinant tissue factor were examined in the clotting assays on microplate. The clotting assay was initiated by adding 80 μL of tissue factor in 25 mM $CaCl_2$ to 40 μL plasma. As shown in **Figure 6,** as the concentration of tissue factor is increased, clotting time was decreased from 600 seconds to less than 150 seconds. The relipidated form of tissue factor is much more effective in the initiation of clotting assay with DE_{50} of about 2.5 ng/mL, while the nonlipidated form is much less effective and clotting time obtained is much longer than 300 seconds even at a concentration of 50 ng/mL.

EXAMPLE 8

Dose-Dependent Prolongation of Tissue Factor-Initiated Clotting Time on Enoxaparin and Heparin Derivatives

The sensitivity of recombinant tissue factor initiated clotting assay to the blood concentration of enoxaparin was further examined. The tissue factor (relipidated) at a concentration of 2.5 ng/mL was used to initiate clotting assay. The clotting time was plotted against the concentration of enoxaparin added to the plasma. It was shown that the clotting assay initiated by relipidated tissue factor is very similar to the diluted prothrombin time, and is just as sensitive to blood enoxaparin (**Figure 7**). The clotting time was significantly prolonged when the plasma concentration of enoxaparin is only 1 μg/mL. Again, there is a linear relationship between the prolongation of clotting time and the concentration of enoxaparin in plasma between 1 μg/mL and 10 μg/mL. The sensitivity of this assay to blood concentration of heparin derivative (Mr. = 3000) was also examined. Similar to what was seen in the diluted prothrombin time, clotting time is significantly prolonged when the concentration of heparin derivative (Mr. = 3000 daltons) in plasma is higher than 1 μg/mL.

The foregoing is illustrative of the present invention, and is not to be taken as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That which is claimed is:

1. A method for determining the concentration of low molecular weight heparin in a blood plasma sample, comprising:

(a) adding a dilute thromboplastin solution to a blood plasma sample; and then

(b) measuring the time to clot formation in said blood plasma sample;

wherein said dilute thromboplastin solution is diluted prior to said adding step so that the time to clot formation in said blood plasma sample is at least 100 seconds when said blood plasma sample contains at least 1 µg/ml of low molecular weight heparin.

2. A method according to claim 1, wherein said measuring step is followed by the step of quantitatively determining the amount of low molecular weight heparin in said blood plasma sample from said measured time to clot formation.

3. A method according to claim 1, wherein said blood plasma sample contains an anticoagulant.

4. A method according to claim 1, wherein said thromboplastin solution contains calcium ion in an amount sufficient to activate said thromboplastin.

5. A method according to claim 1, wherein said thromboplastin solution and said blood plasma sample are warmed to a temperature of about 37°C prior to said adding step.

6. A method according to claim 1, wherein said measuring step is carried out with a fibrometer.

7. A method according to claim 1, wherein said measuring step is carried out on a microplate.

8. A method according to claim 1, wherein said measuring step is carried out

with an automated clotting machine.

9. A kit for determining the concentration of heparin or low molecular weight heparin in a blood plasma sample, comprising:

(a) a concentrated thromboplastin reagent;

(b) a diluent for said concentrated thromboplastin reagent; and

(c) instructions for combining said diluent and said concentrated thromboplastin solution to provide a dilute thromboplastin solution that yields a time to clot formation, when said diluted thromboplastin solution is combined with a blood plasma sample, of at least 100 seconds when said blood plasma sample contains at least 1 μg/ml of low molecular weight heparin.

10. A kit according to claim 9, wherein said thromboplastin reagent contains calcium ion sufficient to activate said thromboplastin upon reconstitution in said diluent.

11. A method for determining the concentration of low molecular weight heparin in a blood plasma sample, comprising:

(a) adding a dilute recombinant tissue factor solution to a blood plasma sample; and then

(b) measuring the time to clot formation in said blood plasma sample;

wherein said recombinant tissue factor is a relipidated recombinant tissue factor; and wherein said dilute recombinant tissue factor solution is diluted prior to said adding step so that the time to clot formation in said blood plasma sample is at least 100 seconds when said blood plasma sample contains at least 1 μg/ml of low molecular weight heparin.

12. A method according to claim 11, wherein said measuring step is followed by the step of quantitatively determining the amount of low molecular weight heparin in said blood plasma sample from said measured time to clot formation.

13. A method according to claim 11, wherein said blood plasma sample contains

an anticoagulant.

14. A method according to claim 11, wherein said recombinant tissue factor solution contains calcium ion in an amount sufficient to activate said recombinant tissue factor.

15. A method according to claim 11, wherein said recombinant tissue factor is mammalian tissue factor.

16. A method according to claim 11, wherein said recombinant tissue factor solution and said blood plasma sample are warmed to a temperature of about 37°C prior to said adding step.

17. A method according to claim 11, wherein said measuring step is carried out with a fibrometer.

18. A method according to claim 11, wherein said measuring step is carried out on a microplate.

19. A method according to claim 11, wherein said measuring step is carried out with an automated clotting machine.

20. A kit for determining the concentration of heparin or low molecular weight heparin in a blood plasma sample, comprising:

(a) a concentrated recombinant tissue factor reagent, wherein said tissue factor is relipidated tissue factor;

(b) a diluent for said concentrated tissue factor reagent; and

(c) instructions for combining said diluent and said concentrated tissue factor solution to provide a dilute tissue factor solution that yields a time to clot formation, when said diluted tissue factor solution is combined with a blood plasma sample, of at least 100 seconds when said blood plasma sample

contains at least 1 μg/ml of low molecular weight heparin.

21. A kit according to claim 20, wherein said recombinant tissue factor reagent contains calcium ion sufficient to activate said tissue factor upon reconstitution in said diluent.

22. A kit according to claim 20, wherein said recombinant tissue factor solution is mammalian recombinant tissue factor solution.

23. A method for determining the concentration of heparin in a blood plasma sample, comprising:

(a) adding a dilute thromboplastin solution to a blood plasma sample; and then

(b) measuring the time to clot formation in said blood plasma sample;

wherein said dilute thromboplastin solution is diluted prior to said adding step so that the time to clot formation in said blood plasma sample is at least 100 seconds when said blood plasma sample contains at least 1 μg/ml of low molecular weight heparin.

24. A method according to claim 23, wherein said measuring step is followed by the step of quantitatively determining the amount of heparin in said blood plasma sample from said measured time to clot formation.

25. A method according to claim 23, wherein said blood plasma sample contains an anticoagulant.

26. A method according to claim 23, wherein said thromboplastin solution contains calcium ion in an amount sufficient to activate said thromboplastin.

27. A method according to claim 23, wherein said thromboplastin solution and said blood plasma sample are warmed to a temperature of about 37°C prior to said adding step.

28. A method according to claim 23, wherein said measuring step is carried out with a fibrometer.

29. A method according to claim 23, wherein said measuring step is carried out on a microplate.

30. A method according to claim 23, wherein said measuring step is carried out with an automated clotting machine.

31. A method for determining the concentration of heparin in a blood plasma sample, comprising:

(a) adding a dilute recombinant tissue factor solution to a blood plasma sample; and then

(b) measuring the time to clot formation in said blood plasma sample;

wherein said recombinant tissue factor is a relipidated recombinant tissue factor; and wherein said dilute recombinant tissue factor solution is diluted prior to said adding step so that the time to clot formation in said blood plasma sample is at least 100 seconds when said blood plasma sample contains at least 1 μg/ml of low molecular weight heparin.

32. A method according to claim 31, wherein said measuring step is followed by the step of quantitatively determining the amount of heparin in said blood plasma sample from said measured time to clot formation.

33. A method according to claim 31, wherein said blood plasma sample contains an anticoagulant.

34. A method according to claim 31, wherein said recombinant tissue factor solution contains calcium ion in an amount sufficient to activate said recombinant tissue factor.

35. A method according to claim 31, wherein said recombinant tissue factor is mammalian tissue factor.

36. A method according to claim 31, wherein said recombinant tissue factor solution and said blood plasma sample are warmed to a temperature of about 37°C prior to said adding step.

37. A method according to claim 31, wherein said measuring step is carried out with a fibrometer.

38. A method according to claim 31, wherein said measuring step is carried out on a microplate.

39. A method according to claim 31, wherein said measuring step is carried out with an automated clotting machine.

FIG. 1

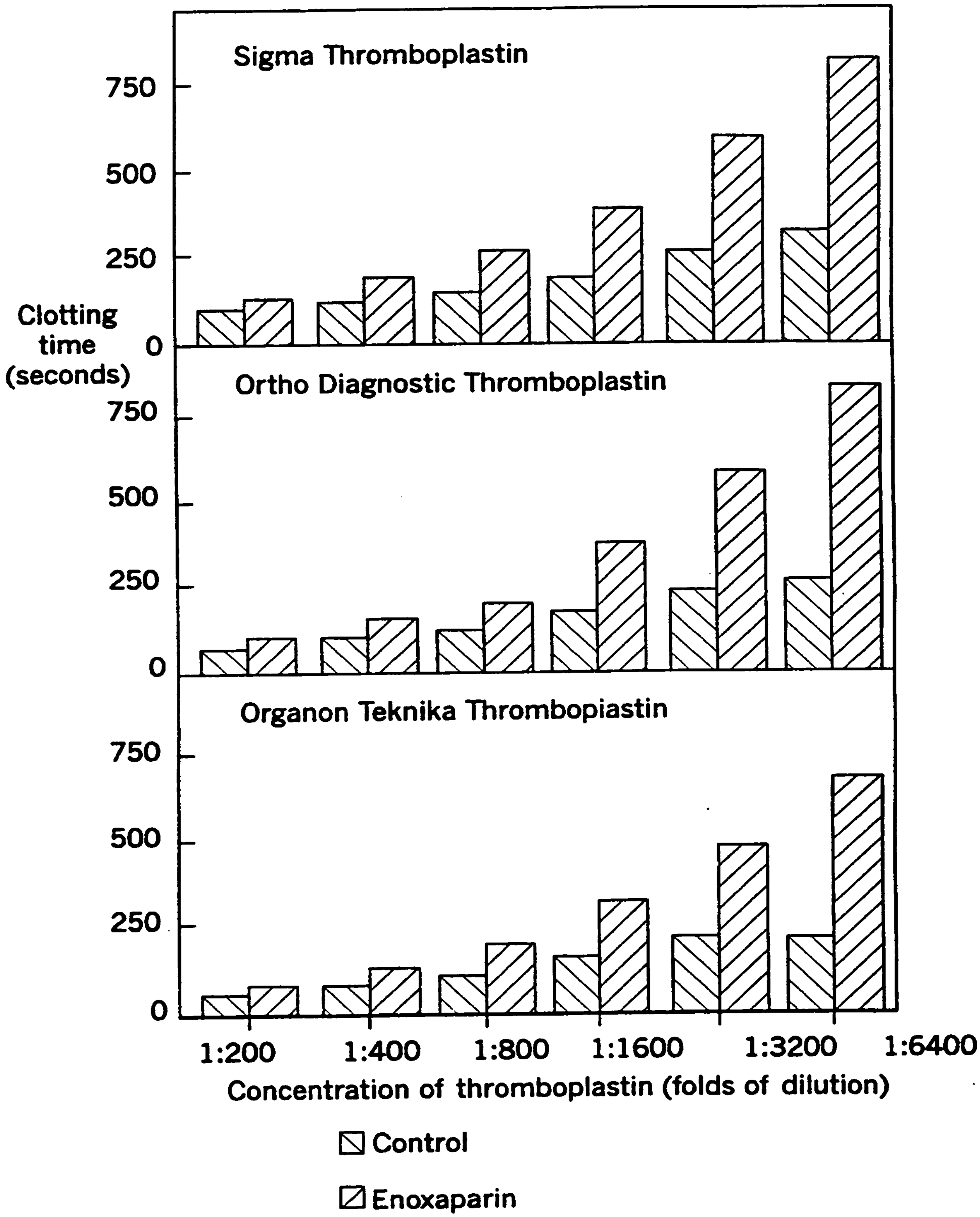

Sigma Thromboplastin
Ortho Diagnostic Thromboplastin
Organon Teknika Thrombopiastin
Clotting time (seconds)
0
250
500
750
1:200
1:400
1:800
1:1600
1:3200
1:6400
Concentration of thromboplastin (folds of dilution)
Control
Enoxaparin

FIG. 2A

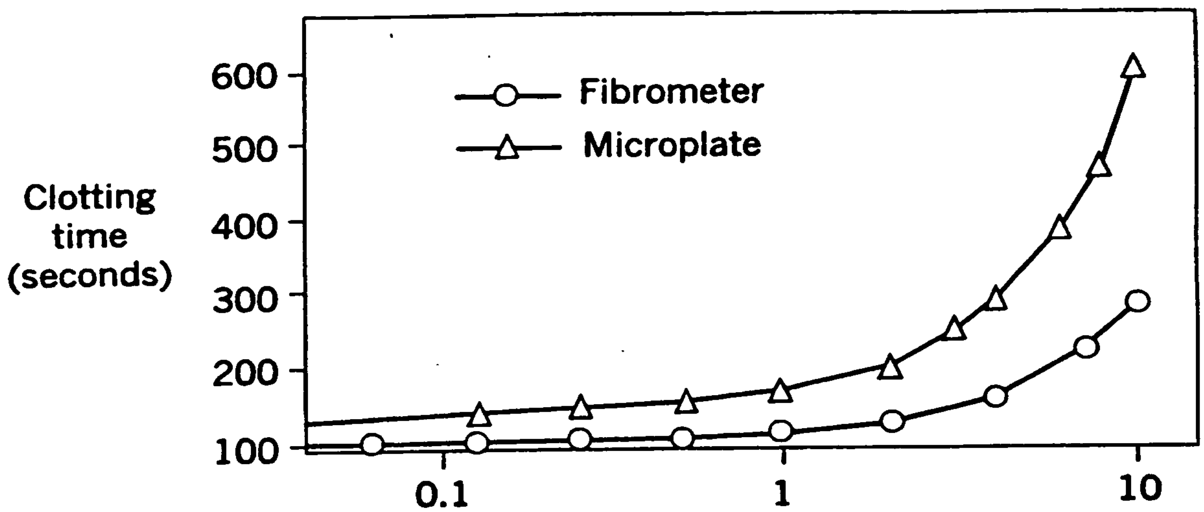

Fibrometer
Microplate
Clotting time (seconds)
600
500
400
300
200
100
0.1
1
10

FIG. 2B

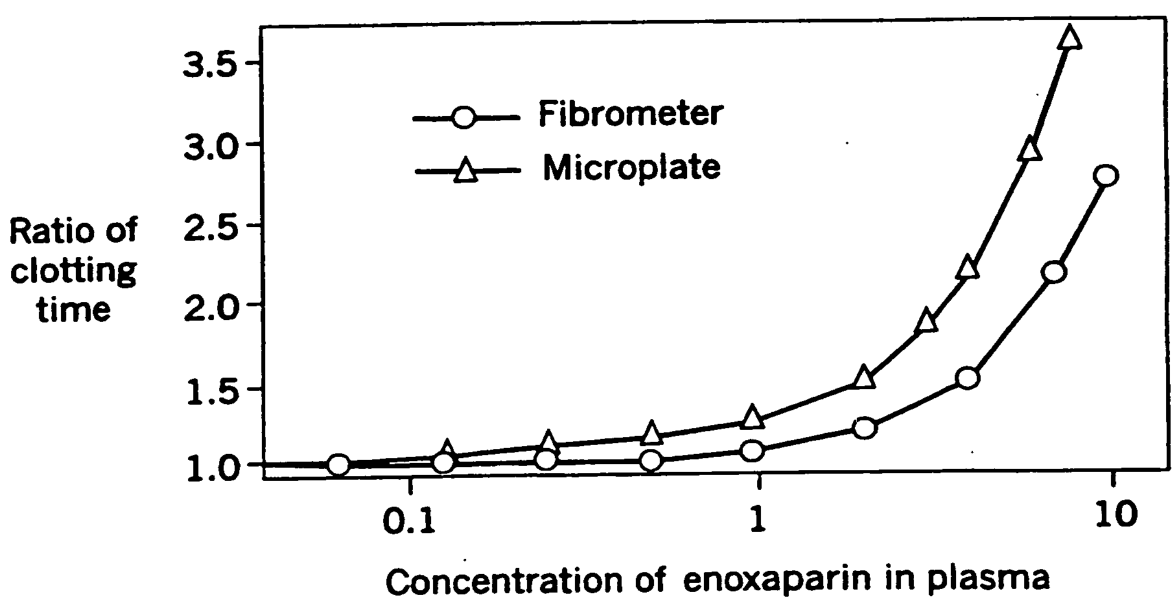

Fibrometer
Microplate
Ratio of clotting time
3.5
3.0
2.5
2.0
1.5
1.0
0.1
1
10
Concentration of enoxaparin in plasma

FIG. 3A

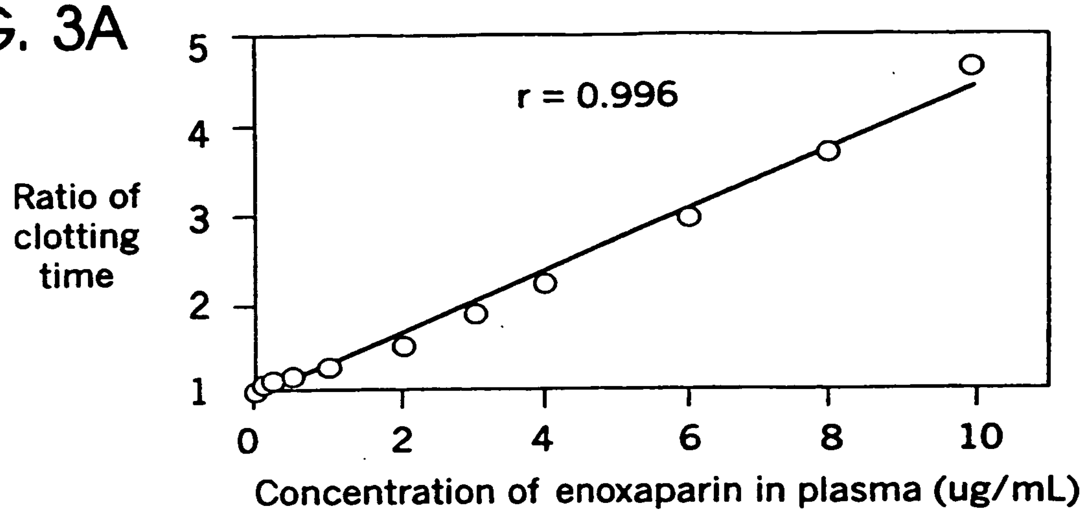


FIG. 3B

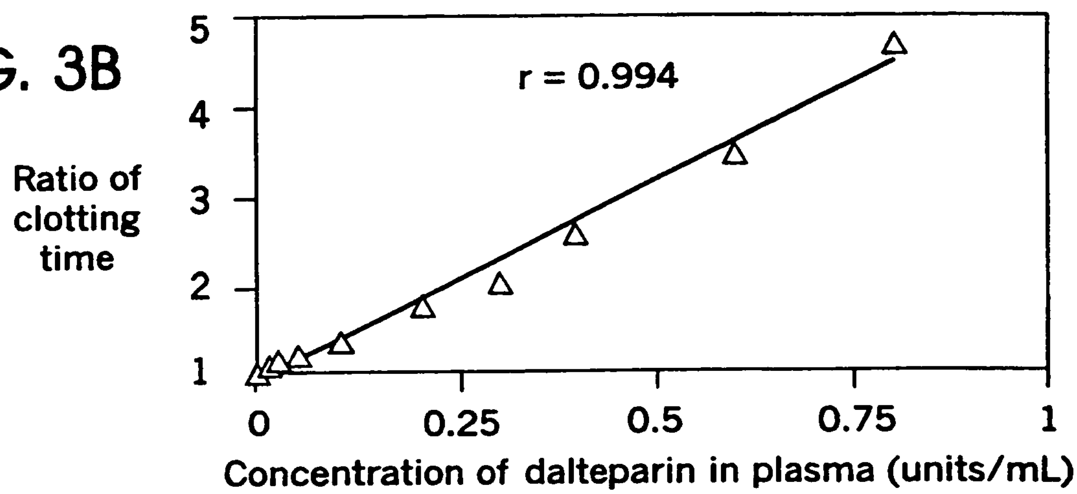


FIG. 3C

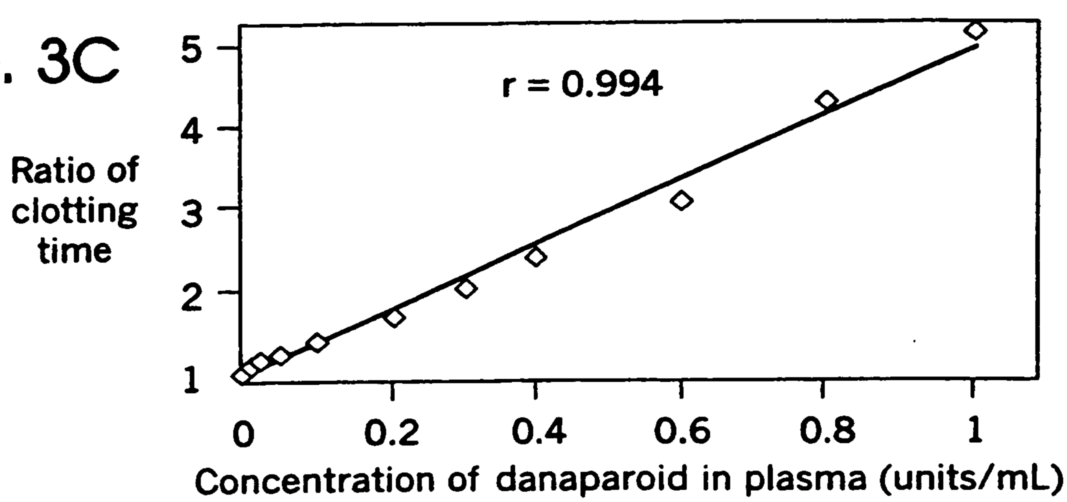


FIG. 4

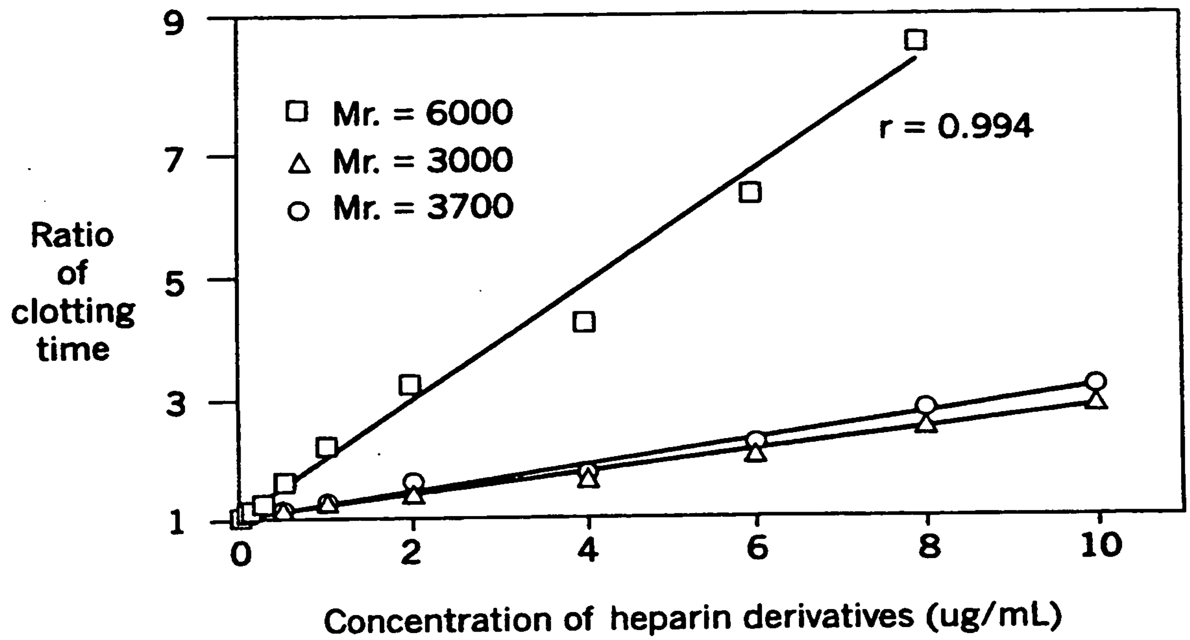

Mr. = 6000
Mr. = 3000
Mr. = 3700
r = 0.994
Ratio of clotting time
Concentration of heparin derivatives (ug/mL)
9
7
5
3
1
0
2
4
6
8
10

FIG. 5

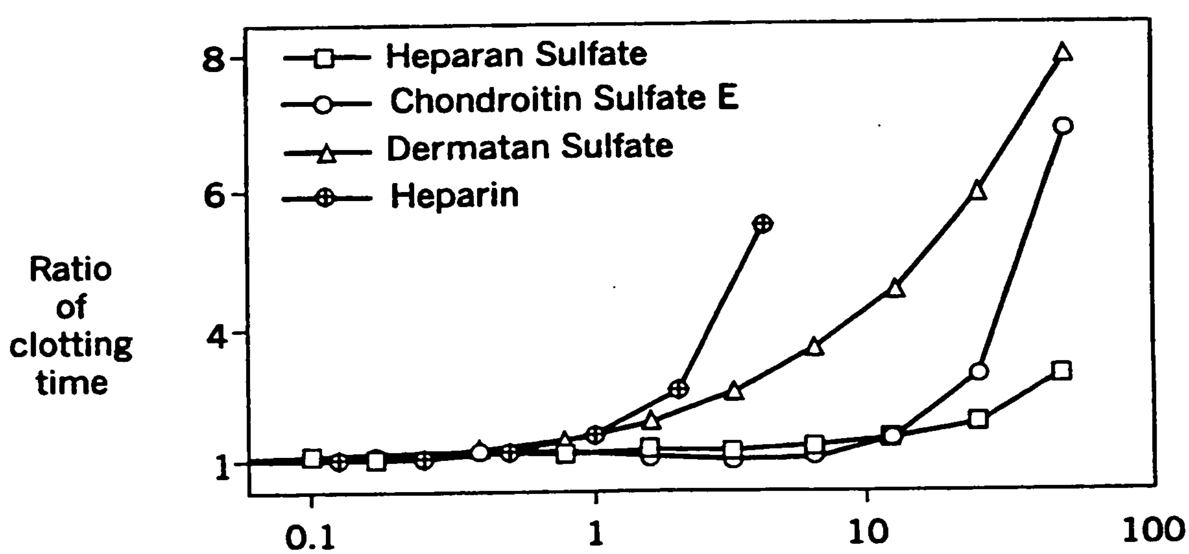

Heparan Sulfate
Chondroitin Sulfate E
Dermatan Sulfate
Heparin
Ratio of clotting time
8
6
4
1
0.1
1
10
100

FIG. 6

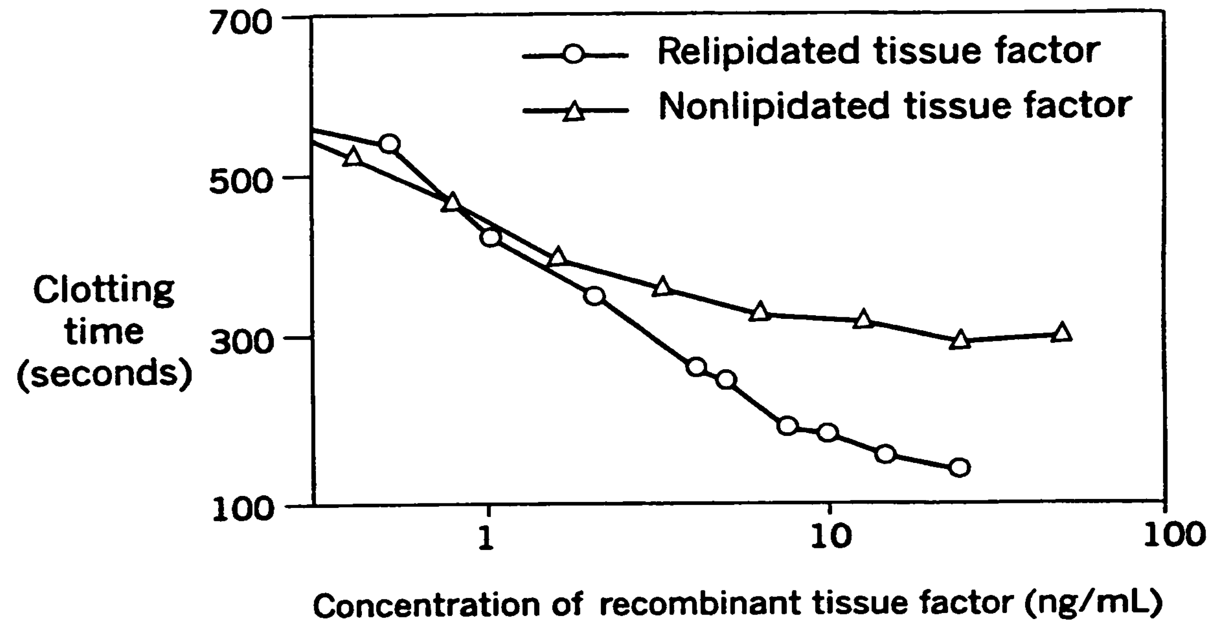
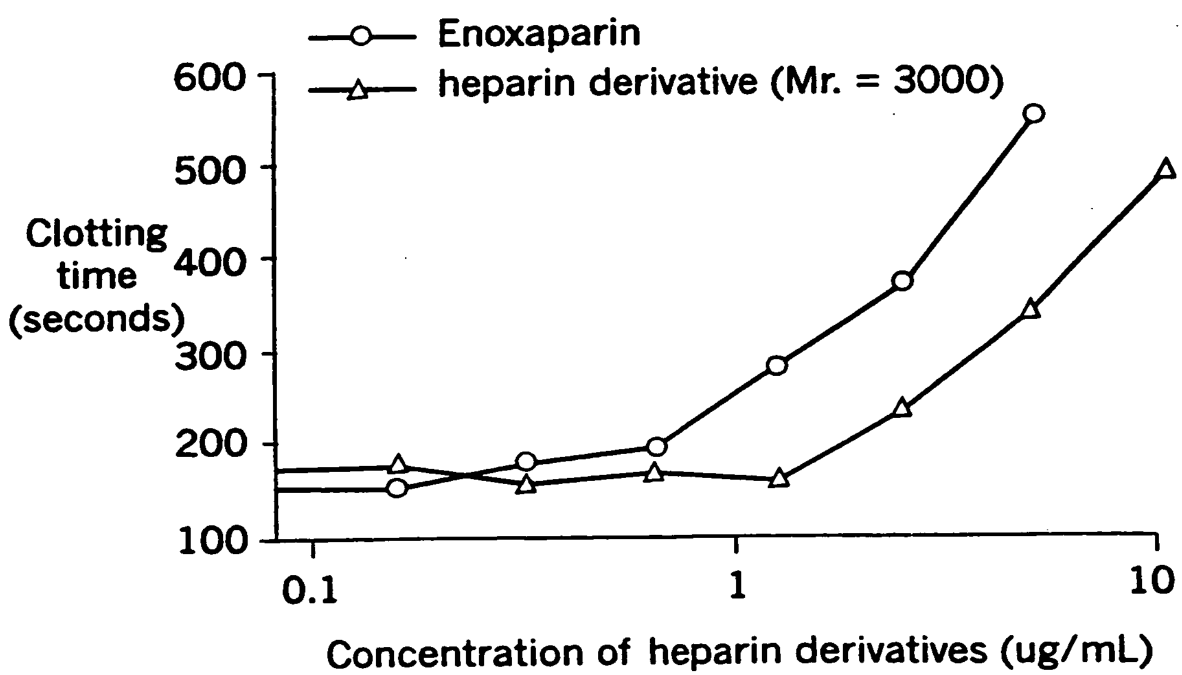

700
500
300
100
Clotting time (seconds)
Relipidated tissue factor
Nonlipidated tissue factor
1
10
100
Concentration of recombinant tissue factor (ng/mL)

FIG. 7


Enoxaparin
heparin derivative (Mr. = 3000)
600
500
400
300
200
100
Clotting time (seconds)
0.1
1
10
Concentration of heparin derivatives (ug/mL)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
G01N 33/86

A3

(11) International Publication Number: WO 99/10746

(43) International Publication Date: 4 March 1999 (04.03.99)

(21) International Application Number: PCT/US98/17434

(22) International Filing Date: 25 August 1998 (25.08.98)

(30) Priority Data:
08/920,151 26 August 1997 (26.08.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US Not furnished (CON)
Filed on Not furnished

(71) Applicant (*for all designated States except US*): THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; 308 Bynum Hall, CB #4105, Chapel Hill, NC 27599–4105 (US).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): WU, Hai-feng [CN/US]; 101 William White Court, Carrboro, NC 27510 (US).

(74) Agents: SIBLEY, Kenneth, D. et al.; Myers, Bigel, Sibley, & Sajovec, P.A., P.O. Box 37428, Raleigh, NC 27627 (US).

(81) Designated States: AU, CA, JP, KR, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

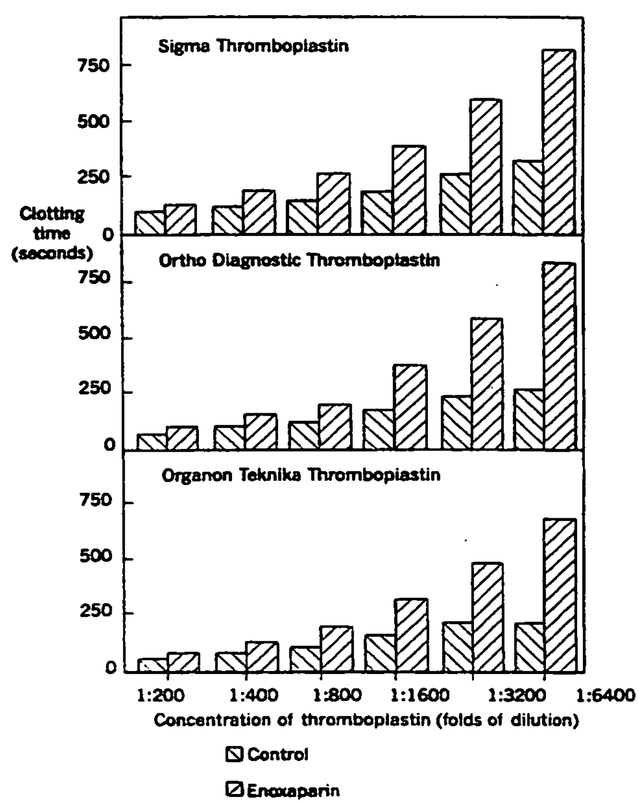
Published
With international search report.

(88) Date of publication of the international search report: 20 May 1999 (20.05.99)

(54) Title: METHOD OF MONITORING BLOOD LOW MOLECULAR WEIGHT HEPARIN AND HEPARIN

(57) Abstract

A method for determining the concentration of low molecular weight heparin or heparin in a blood plasma sample comprises the steps of: (a) adding a dilute thromboplastin (or tissue factor) solution to a blood plasma sample; and then (b) measuring the time to clot formation in said blood plasma sample. In one embodiment, the dilute thromboplastin (or tissue factor) solution is diluted prior to the adding step so that the time to clot formation in said blood plasma sample is at least 100 seconds when said blood plasma sample contains at least 1 μg/ml of low molecular weight heparin.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/17434

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K A61K C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L NORRHEIM, U ABILDGAARD, M L LARSEN, A K LINDAHL: "Involvement of the Extrinsic Pathway in the Activities of Low Molecular Weight Heparins" THROMBOSIS RESEARCH. SUPPLEMENT (14), vol. 14, 1991, pages 19-27, XP002094528	1-10, 23-30
Y	see page 23, line 4; table 1	11-22, 31-39
	see page 24, paragraph 3 see page 25, line 5; table 2 ---	
Y	US 5 625 036 A (HAWKINS PAMELA L ET AL) 29 April 1997 cited in the application see the whole document ---	11-22, 31-39
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 1999	15/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hart-Davis, J

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 98/17434

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 511 075 A (RHONE POULENC RORER SA) 28 October 1992 see page 5, line 57 - line 58	1-39
A	O NORDFANG, H I KRISTENSEN, S VALENTIN, P OSTERGAARD, J WADT: "The Significance of TFPI in Clotting Assays - Comparison and Combination with Other Anticoagulants" THROMBOSIS AND HAEMOSTASIS, vol. 70, no. 3, 1 September 1993, pages 448-453, XP002094529 see figure 1B	1-39
A	US 4 415 559 A (SUZUKI SUGURU ET AL) 15 November 1983 see table 1	1-39
A	EP 0 219 400 A (UNIV COMPIEGNE) 22 April 1987 see page 34, line 1 - line 19 see page 38, line 30 - page 40, line 4; tables 3,4	1-39

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte	onal Application No
PCT/US 98/17434

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 5625036	A	29-04-1997	AT	163768	T	15-03-1998
			AU	663343	B	05-10-1995
			AU	2769292	A	03-05-1993
			CA	2097199	A	05-04-1993
			DE	69224622	D	09-04-1998
			DE	69224622	T	05-11-1998
			EP	0565665	A	20-10-1993
			ES	2115679	T	01-07-1998
			JP	6505562	T	23-06-1994
			WO	9307492	A	15-04-1993
EP 0511075	A	28-10-1992	FR	2675806	A	30-10-1992
			AU	1748592	A	17-11-1992
			EP	0581846	A	09-02-1994
			WO	9218544	A	29-10-1992
			JP	6506968	T	04-08-1994
			MX	9201846	A	01-02-1993
			NZ	242431	A	27-04-1994
			US	5707973	A	13-01-1998
			US	5721357	A	24-02-1998
US 4415559	A	15-11-1983	JP	1502468	C	28-06-1989
			JP	57134419	A	19-08-1982
			JP	63053970	B	26-10-1988
			EP	0058397	A	25-08-1982
EP 0219400	A	22-04-1987	FR	2587707	A	27-03-1987